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Purpose: The stability of the heme-globin interaction of chemically modified human hemoglobin was tested by measuring rates of heme exchange from methemoglobin to human serum albumin.

Protocol: Heme exchange was measured by rapid-scanning spectrophotometry, adapted from the procedure of Benesch. Experimental conditions were 50 μM (in heme) hemoglobin (Hb) and 50 μM albumin in Tris buffer, pH 9.0, at 20°C. Hemoglobins tested were: HbA_o, Hb cross-linked between the β subunits (β-β) with either 2-nor-2-formylpyridoxal 5'-phosphate or 3,5-di(bromosalicyl)fumarate (DBBF), Hb cross-linked between the α subunits (α-α) with DBBF, and pyridoxalated Hb polymerized (poly) with glycolaldehyde or glutaraldehyde. Time courses were fitted to a biexponential equation to determine the rates and amplitudes of the reactions

Results: Reaction rates fell into two groups: (1) HbA_o and the site-specifically cross-linked Hbs, with biexponential rates of 0.02 and 0.004 min^{-1}, and (2) polymerized Hbs, with 10-fold higher rates at 0.5 and 0.03 min^{-1}. The amplitudes of the reaction depended upon the

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specific modification: [illegible] Hbs [illegible] Hb = glycolaldehyde poly Hb \glutaraldehyde poly Hb $\leq$ HbA_o.

Conclusions: Polymerization alters the heme-globin interaction, such that heme exchange occurs faster. Tetrameric cross-linking does not alter the rate compared with HbA_o, but does decrease the overall amplitude of the reaction, suggesting that the probability of heme exchange is less.

A COMPARISON OF RATES OF HEME EXCHANGE: SITE-SPECIFICALLY CROSS-LINKED *VERSUS* POLYMERIZED HUMAN HEMOGLOBINS

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ABSTRACT

The stability of the heme-globin interaction of chemically modified human hemoglobin (Hb) was tested by measuring rates of heme loss from methemoglobin. Heme transfer from methemoglobin to human serum albumin was measured by rapid-scanning spectrophotometry, and the resulting absorption matrices were analyzed by singular value decomposition. Unmodified human HbA_0, hemoglobin cross-linked between β subunits with either 2-nor-2-formylpyridoxal 5′-phosphate or 3,5-(dibromosalicyl)fumarate (DBBF), hemoglobin cross-linked between α subunits with DBBF, and pyridoxalated hemoglobin polymerized with either glycolaldehyde or glutaraldehyde were tested. Initial rates were evaluated by fitting the time courses to a biexponential equation using a matrix least squares curve-fitting algorithm. Reaction rates fell into two classes: (1) HbA_0 and the site-specifically cross-linked hemoglobins, with biphasic rates of heme loss of 0.02 and 0.004 min^{-1}, and (2) polymerized hemoglobins, with 10-20-fold higher rates at 0.5 and 0.03 min^{-1}. The total fitted amplitudes of the reaction depended upon the specific modification: ββ-cross-linked Hbs < αα-cross-linked Hb ≈ glycolaldehyde polymerized Hb < glutaraldehyde polymerized Hb < HbA_0.

INTRODUCTION

Designing hemoglobin-based red blood cell substitutes requires identifying the fundamental properties of oxygen transport by red blood cells and conferring those properties on cell-free solutions of hemoglobin without inflicting untoward side-effects. Not everything about red cell function is known, but a few important properties are obvious. (1) Red blood cells carry hemoglobin at high intracellular concentrations, providing high O_2-carrying capacity and favoring the tetrameric form of hemoglobin over dimer formation. (2) Human red blood cells produce 2,3-diphosphoglycerate (2,3-DPG) during their normal glycolytic metabolism; 2,3-DPG binds to hemoglobin and lowers oxygen affinity, thus increasing the efficacy of oxygen release to tissues. (3) Human red blood cells produce an intracellular methemoglobin reductase enzyme system that minimizes the amount of methemoglobin inside normal red blood cells to ≤3-5% of total cellular hemoglobin.

The first two properties have been addressed in the design of hemoglobin-based blood substitutes. Both prevention of hemoglobin tetramer dissociation and alteration of intrinsic oxygen affinity can be achieved by chemical cross-linking or genetic manipulations [*for a review, see ref.* 1]. However, the third property listed above has not been met. Autooxidation of native, chemically modified or genetically altered hemoglobins has not yet been subject to control by design. And unfortunately, the rate of autooxidation of ferrous (Fe^{2+}) oxyhemoglobin to ferric (Fe^{3+}) methemoglobin has been found so far to be inversely proportional to O_2 affinity [2].

Methemoglobin formation is doubly disadvantageous. (1) It does not bind O_2, and (2) oxidation is the first step in hemoglobin denaturation. Ferric heme has a reduced affinity for globin [3], leading to heme loss and apoprotein precipitation. This may be particularly undesirable in cell-free hemoglobin solutions, because in the absence of the red blood cell membrane barrier, other

oxidatively sensitive membranes, such as endothelial membranes, become exposed directly to free heme.

To address this issue, we measured rates of heme loss from different methemoglobin derivatives as an indication of the effects of protein modification on the stability of the heme-globin linkage.

MATERIALS AND METHODS

All hemoglobin samples were derived from human blood. Three of the hemoglobin samples used in these experiments were provided by the Letterman Army Institute of Research Hemoglobin Production Facility. These included: hemoglobin A_0 (HbA_0), hemoglobin cross-linked between α subunits ($\alpha\alpha$Hb) by 3,5-(dibromosalicyl)fumarate (DBBF) [4], and glycolaldehyde polymerized pyridoxalated hemoglobin [5]. The polymerized sample was produced in collaboration with Duncan Pepper and Shirley MacDonald of the Scottish National Blood Transfusion Service. Hemoglobin cross-linked between β subunits with 2-nor-2-formylpyridoxal 5 '-phosphate ($\beta\beta$Hb-NFPLP) was provided by Joa Bakker of the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service [*see ref.* 6]. Hemoglobin cross-linked between β subunits by DBBF ($\beta\beta$Hb-DBBF) [7] and glutaraldehyde polymerized pyridoxalated hemoglobin [8] were provided by Angelo Zegna of the Blood Research Division, Letterman Army Institute of Research.

The heme exchange assay was adapted from the method of Benesch and Kwong [9]. Methemoglobin was prepared by reacting hemoglobin with a 1.2-fold excess of potassium ferricyanide to heme at room temperature in 0.1 M bis-Tris buffer, pH 7.0. Ferro- and ferricyanide were removed by gel filtration on a Sephadex G-25 column equilibrated with 50 mM bis-Tris buffer, pH 7.5, containing 0.1 M Cl^-.

The assay conditions were as follows: 50 μM (in heme) methemoglobin and 50 μM human serum albumin (HSA), 0.25 M Tris buffer, pH 9.0, and

20°C. The reaction was initiated by the addition of HSA. A rapid-scanning spectrophotometer (LT Quantum 1200), scanning from 400 to 800 nm in 200 ms, was used to monitor the reaction as heme was lost from methemoglobin and bound to HSA. For each time point, four spectra were collected and averaged into a single spectrum with a time resolution of 0.8 s. Data collection was under computer control to provide exact intervals in time between spectral measurements from 0.5-10 min, depending on how quickly the reaction occurred.

For data analysis, spectra from 480 to 650 nm at 1-nm intervals were combined in a single absorbance matrix (**A**) with a total of 171 wavelengths, or rows. The number of columns in **A** were determined by the number of time points in the reaction. Two matrix procedures were used for analysis of **A**: singular value decomposition (SVD) and matrix least squares. A brief description of these methods is given here. A more rigorous discussion can be found in Vandegriff and Shrager [10].

SVD determines the number, or rank, r, of optical species undergoing a transition in **A** by decomposing the **A** matrix into three other matrices,

$$\mathbf{A} = \mathbf{USV}^{\mathrm{T}} \tag{1}$$

where the columns in **U** are linear combinations of the independent spectra of each component in **A**, the columns of **V** are linear combinations of the transitions of each component in **A**, and **S** gives the singular values of **A**. For columns i where $i > r$, **U** column i and **V** column i contribute negligibly to the signal in **A**, and the minimal form of the data in **A** can be created from the columns of **U**, **S**, and **V** that contain signal.

The matrix **A** also is described by two other matrices,

$$\mathbf{A} = \mathbf{DF}^{\mathrm{T}} \tag{2}$$

where the columns in **D** contain the spectra that are changing, and the columns in **F** give the transition curves for the spectra in **D**. **F** can be fitted for by predicting a model for the transitions in **A** and using matrix least squares to

adjust the parameters in **F** by curve fitting to minimize the residual sum of squares for $\mathbf{A} - \mathbf{DF}^T$, where **A** is known. To model **F** for these reactions, a biexponential equation was used with equal amplitudes for the two phases, *i.e.*,

$$k_{obs} = 0.5A_T(e^{-kft} + e^{-kst}) \quad (3)$$

where k_{obs} is the rate of methemoglobin disappearance, A_T is total change in amplitude, and k_f and k_s are, respectively, the fast and slow rate constants.

RESULTS

The heme exchange reaction was measured for purified HbA_0, three site-specifically cross-linked human hemoglobin tetramers, and two polymerized pyridoxalated human hemoglobins. The reaction of DBBF with oxyhemoglobin cross-links the tetramer primarily between βLys(82) residues in the β pocket [7], with a smaller percentage cross-linked between β_1Lys(82) and, most likely, β_2Lys(144) [11]. NFPLP cross-links deoxyhemoglobin between the N-terminal amino group of one β subunit [*i.e.*, β_1Val(1)] and β_2Lys(82) of the opposite β subunit [12]. The reaction of deoxyhemoglobin with DBBF cross-links the tetramer between αLys(99) residues in the central cavity of the hemoglobin molecule [13]. The samples of polymerized pyridoxalated hemoglobin used in this study were prepared with the multifunctional aldehyde, glutaraldehyde (polyGlutar) or with the monoaldehyde, glycolaldehyde (polyGlycol).

The spectral change during heme exchange from $metHbA_0$ to HSA is shown in **Figure 1*A***. As can be seen from the experimental spectra compared with the spectrum of methemalbumin (dashed line, **Figure 1*A***), the reaction did not go to completion. Under these conditions, when apohemoglobin becomes ~50% of the total, protein denaturation begins to occur, and the spectral baseline shifts.

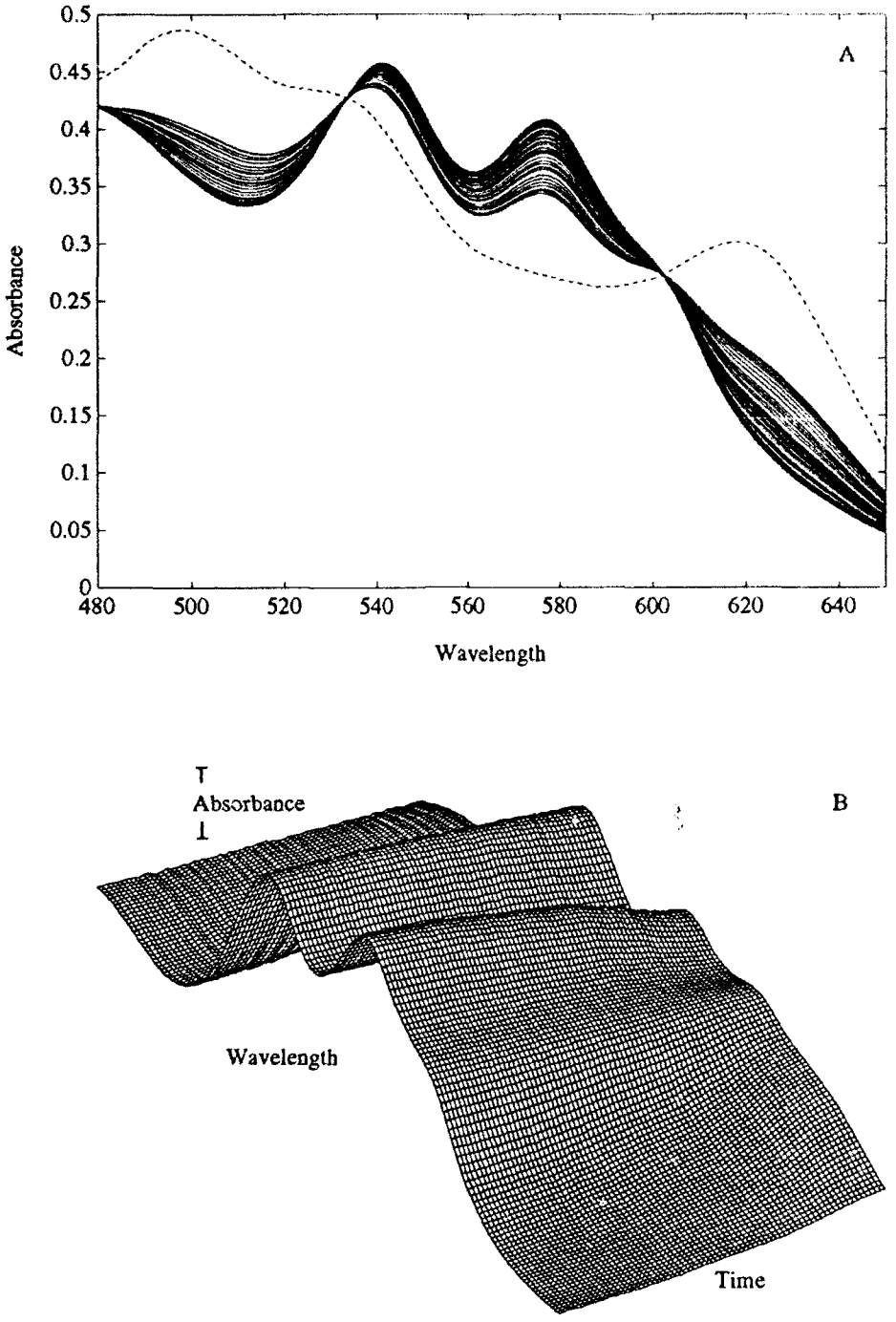

Figure 1. (*A*) ***Solid lines*, absorption spectra during heme exchange from metHbA$_0$ to HSA.** ***Dashed line*, methemalbumin.** (*B*) **Matrix A.**

The three-dimensional absorbance matrix **A** for the experiment with HbA_0 is shown in **Figure 1*B***. Wavelength is shown along the x-axis from 480 to 650 nm, absorbance is presented along the y-axis, and time is represented by the z-axis. The S matrix from SVD of **A** was used to estimate the rank of **A** by plotting $\log_{10}(s_{i,i})$ *versus i* (**Figure 2**). The number of components that stand out above the declining smooth curve provides an estimate of rank. It is difficult to determine rank exactly, but it is clear that beyond the 3rd-5th component, a signal is difficult to resolve above random noise. This was found to be the case for all of the hemoglobin samples. As a result, minimal matrices **A** were constructed from each of the hemoglobin data sets from the top five components of SVD matrices **U**, **S**, and **V**.

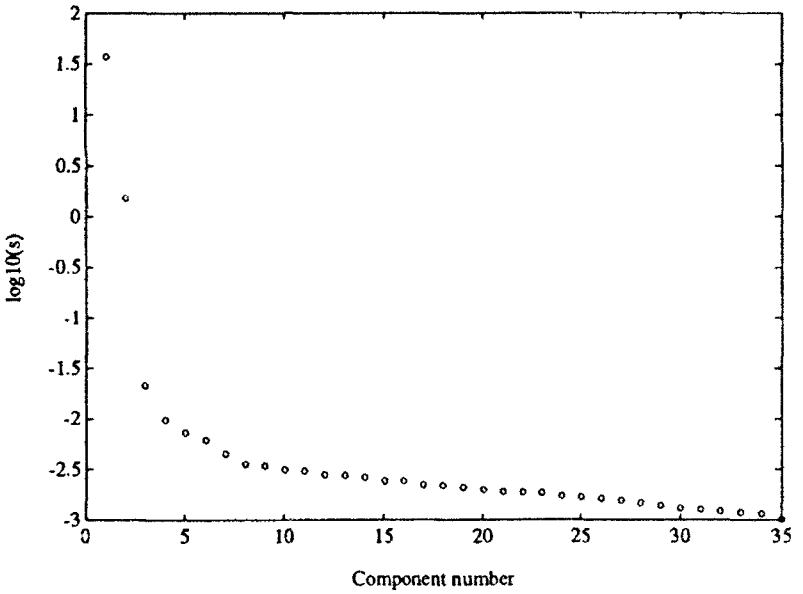

Figure 2. Evaluation of the rank of A.

A matrix least squares algorithm was used to fit for the amplitudes and rates of the reactions. The non-polymerized tetrameric hemoglobin samples could be fit to either mono- or biexponential expressions, but the residual sum of squares were ~2-fold less using the biexponential function in Eq. (3). The polymerized hemoglobin time courses could not be fit to a single exponential function.

The amplitudes of the two phases in the biexponential expression were either constrained to 50% of the total amplitude [as in Eq. (3)] or allowed to float. In either case, the final best-fit amplitudes for the two kinetic phases for all of the modified hemoglobins were ~50% of the total. For HbA_0, the fractional amplitudes converged to 30% of the total for the fast phase and 70% for the slow phase, but this fit was not improved over the fit to Eq. (3). Thus, Eq. (3) was used for all of the final curve-fitting analyses.

Matrix least squares was performed by setting **F** = Eq. (3) and allowing the parameters A_T, k_f, and k_s to be adjusted to minimize the residuals. The columns in **F** represent the time courses for the disappearance of methemoglobin and the appearance of methemalbumin. The best-fit parameters for these six time courses are given in **Table I**. The corresponding time courses for the disappearance of methemoglobin are presented in **Figure 3**.

Table I

INITIAL RATES OF HEME EXCHANGE[a]

Hb	Amplitude (total)	k_f (min^{-1})	k_s (min^{-1})
CLASS I			
ββ (DBBF)	0.16	0.02	0.004
ββ (NFPLP)	0.18	0.03	0.005
αα (DBBF)	0.39	0.02	0.004
A_0	0.67	0.02	0.004
CLASS II			
polyGlycol	0.38	0.42	0.031
polyGlutar	0.49	0.49	0.035

[a]Conditions: [metHb] = 50 μM (in heme); [HSA] = 50 μM; Tris buffer, pH 9.0; 20°C.

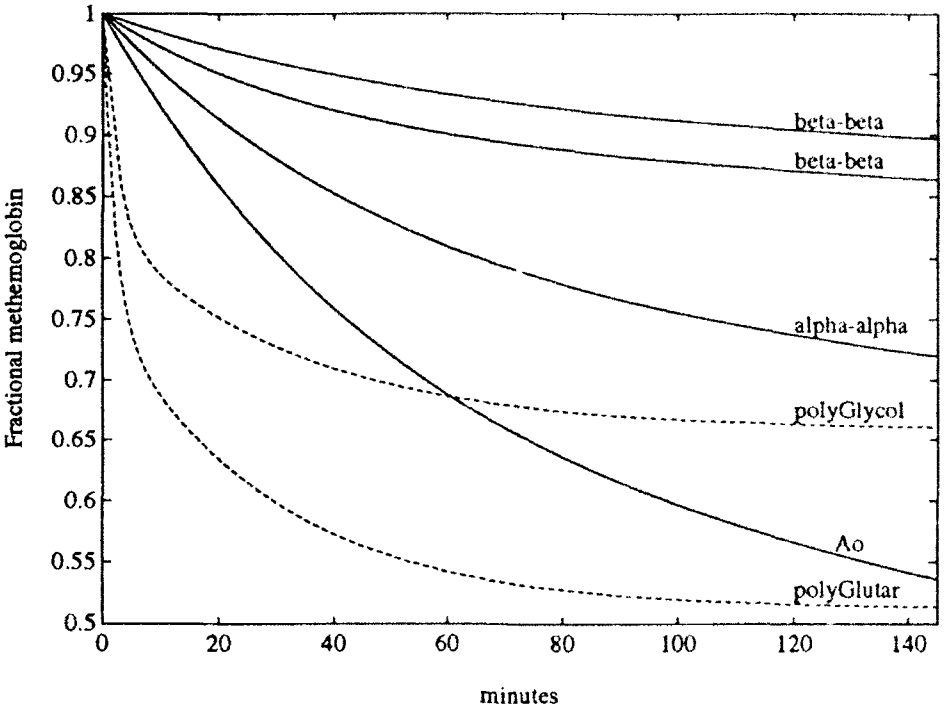

Figure 3. Matrix least squares best-fit curves for the disappearance of methemoglobin: F column 1. Class I, shown in *solid lines* from top to bottom: ββ-cross-linked Hb (DBBF), ββ-cross-linked Hb (NFPLP), αα-cross-linked Hb (DBBF), and HbA_0. **Class II**, shown in *dashed lines* from top to bottom: glycolaldehyde polymerized pyridoxalated Hb (polyGlycol) and glutaraldehyde polymerized pyridoxalated Hb (polyGlutar).

Initial rates of the reaction for these six hemoglobin samples fall into two distinct classes: (**Class I**) unmodified or cross-linked tetrameric human hemoglobin and (**Class II**) polymerized human hemoglobin. The initial rates for the reactions within a class are the same, with variations only in total amplitude. **Class I** hemoglobins show fast and slow rates of 0.02-0.03 and 0.004-0.005 min^{-1}, respectively. **Class II** hemoglobins show respective rates at least 10-fold greater, *i.e.*, 0.4-0.5 and 0.03-0.04 min^{-1}. The total fitted amplitudes for the reactions were for **Class I**: ββHb < ααHb < HbA_0, and for **Class II**: glycolaldehyde polymerized Hb < glutaraldehyde polymerized Hb.

DISCUSSION

The success of modified hemoglobin solutions as red cell substitutes will depend on the efficacy of oxygen transport balanced against toxic effects. Some of the toxic reactions of cell-free hemoglobin solutions have been resolved. Renal injury can be alleviated by preventing dimer formation and excretion. Pyrogenic effects of red cell stromal phospholipids and/or endotoxin contamination can be prevented by following rigorous purification strategies. The causes and prevention of other documented toxicities are still being debated [*for reviews, see refs.* 1 and 14]. Hypertension and free radical damage are two key areas under study. Free radicals are of primary concern because oxygen-free radicals are formed during hemoglobin oxidation. In the absence of methods to prevent autooxidation, oxidative by-products must be dealt with in the overall design of hemoglobin-based blood substitutes.

Hemoglobin subunits have unequal affinities for heme. β subunits lose their hemes ~10-fold faster than α subunits [3]. Therefore, with unmodified HbA_0 and site-specifically cross-linked hemoglobin tetramers, heme loss from β globin likely provided the majority of the observed absorption change. Release of heme from α subunits is more difficult to measure because of globin precipitation [9, 15]. The smaller rate found in these experiments may correspond to heme release from α subunits, but the overall extent of the reaction does not allow a truly definitive interpretation at this time.

Our results suggest that site-specifically cross-linking hemoglobin does not alter the heme-globin interaction in a way that affects rates of heme loss. In contrast, polymerization causes a marked increase in rates of heme loss. This is consistent with a Mössbauer spectroscopic study of hemoglobin that showed that the heme pocket is opened up by polymerization [16]. As a result, polymerized hemoglobins may be more susceptible to oxidation and heme loss.

The fast rate for the polymerized hemoglobins is 10-20-times faster than the fast rate for the non-polymerized hemoglobins (0.4 *versus* 0.02-0.03 min^{-1}),

and the slow rate for the polymerized samples is equal to the fast rate for the non-polymerized samples. Thus, two explanations are possible: (1) the two rates for polymerized hemoglobin may correspond to heme loss by both subunits, with both rates being markedly faster than the corresponding rates for non-polymerized samples, or (2) the fast phase may correspond to destabilized heme linkages in 50% of the polymerized monomers, while the remaining slower 50% of the reaction corresponds to a normal rate of β-subunit heme loss as in the non-polymerized hemoglobins. Because each kinetic phase contributes equally to the total amplitude of the reaction, the first explanation, *i.e.*, that the two rates reflect α- and β-subunit heme loss, is more probable.

The different amplitudes for the reactions within each class suggest that these modifications affect the probability of the reactions more than the initial rates. HbA_0 gave the largest amplitude. As suggested by Benesch and Kwong [9], this is likely because HbA_0 dissociates into dimers, which are less stable, whereas, cross-linked hemoglobins do not dissociate. The different amplitudes observed for the polymerized hemoglobins may be the result of the polymerizing agents. Glycolaldehyde is a monoaldehyde that is shorter and not as reactive as the multifunctional glutaraldehyde; the glycolaldehyde reaction is more controlled and forms fewer intermolecular cross-links [5].

Our assay conditions do not reflect *in vivo* pH or temperature but are necessary for the spectral measurements and apoprotein stability. A new method, using a mutant apomyoglobin that becomes green upon heme binding, is being developed (*see*, J.S. Olson's chapter, this volume). Methods to improve the stability of the apoproteins so that the reaction can be followed to completion at physiological pH and temperature will provide more definitive analyses of the reaction and possibly better extrapolation to clinical situations.

Authors' note: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the U.S. Department of the Army or the Department of Defense.

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